A gonad-derived survival signal for vulval precursor cells in two nematode species

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Intercellular cell-survival signals play a major role in animal development [1]. In the nematode *Caenorhabditis elegans*, however, the stereotyped cell deaths that occur reproducibly during development are regulated in a cell-autonomous fashion (or, in a few cases, by a death-inducing signal) [2]. We show here the existence of a cell-survival signal acting on the vulval precursor cells in two nematodes, *Turbatrix aceti* and *Halicephalobus sp.* JB128. In *C. elegans* [3], as in many other nematode species [4–7], ablation of the gonad causes all vulval precursor cells to adopt a default epidermal fate: a gonadal signal is required for the induction of vulval fates. In the nematodes *T. aceti* and *Halicephalobus sp.* JB128, however, we found that ablation of the gonad in the L1 larval stage caused all vulval precursor cells to undergo programed cell death. Thus, in intact *Turbatrix* and *Halicephalobus*, a survival signal from the gonad prevents activation of the cell-death program in vulval precursor cells. Our results demonstrate the existence of intercellular cell-survival signals in nematodes and uncover an evolutionary variation in the role of the gonad in nematode vulval development.

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Results and discussion

Gonad ablation causes vulval precursor cell death in *Turbatrix aceti*

In the vinegar eelworm *T. aceti*, as in other species of the Panagrolaimidae family of nematodes [4,6,7], the vulva develops during the larval L3 and L4 stages from four precursor cells P5.p to P8.p (designated P(5–8).p; see Figure 1). Twelve Pn.p cells, P(1–12).p, are born in the L1 stage and are aligned anteroposteriorly in the ventral cord of the larva. In *Turbatrix*, as in *Panagrolaimus spp.* [6,7], the four anterior cells P(1–4).p die in the L1 stage, shortly after birth, and the four posterior cells P(9–12).p survive and acquire specific, non-vulval fates (Figure 1). Vulval fates of P(5–8).p cells are induced by a gonadal signal in *Panagrellus redivivus* [4] and *Panagrolaimus sp.* PS1732 [7], both members of the Panagrolaimidae family. In these species, as in *C. elegans* [3], a member of the Rhabditidae family, ablation of the gonad in the L1 stage causes all competent cells to adopt a non-vulval, epidermal fate by default.

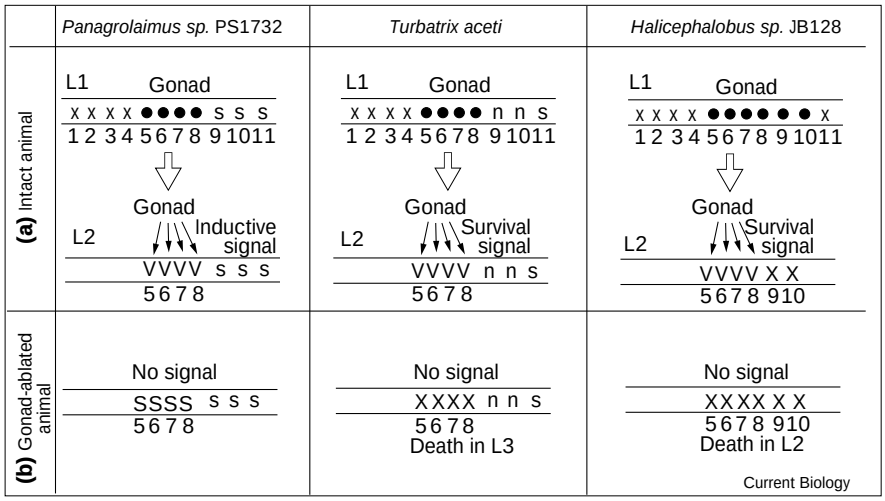
We ablated the gonad in the early L1 stage in *Turbatrix* and found that the vulval precursor cells P(5–8).p died in the L3 stage, at the time when they would normally divide (assessed by the division of the Pn.aap cells, other cells of the ventral cord that divide in the L3 stage around the same time as P(5–8).p, as in *P. redivivus* [4]). The dying cells had the characteristic morphology of cells undergoing programed cell death in nematodes [8] (Figure 2). When only the two germ-line precursors were ablated, vulval lineages were normal, whereas ablation of the somatic precursors alone lead to the death of P(5–8).p. Thus, in *Turbatrix*, the vulval precursor cells normally survive because they receive a survival signal from the somatic gonad.

To determine the timing of this signal, we ablated the gonad at different times during larval development. After ablation of the gonad in the late L1 stage (after the birth of the Pn.p cells) or in the early L2 stage, most of the vulval precursors died. After ablation of the gonad in the late L2 stage, most of the vulval precursors survived and had four vulval progeny (Table 1). Thus, the survival signal is sent to P(5–8).p during the L2 stage, as is the first vulval induction signal in *Panagrolaimus sp.* PS1732 [7]. In animals in which the gonads were ablated early and in which most of the P(5–8).p cells died, the rare survivors adopted a vulval fate. Thus, in *Turbatrix*, cell death and the vulval fate appear to be alternative fates for P(5–8).p. It is possible that the same gonadal signal in the L2 stage prevents cell death and thereby promotes vulval fates in P(5–8).p.

Pn.p cell death in *Halicephalobus sp.*

In a related species, *Halicephalobus sp.* JB128 (family Panagrolaimidae), two temporal waves of Pn.p cell death occur in intact animals. First, P(1–4).p and P11.p die in the L1 stage, shortly after their birth (Figure 1a); then, P9.p and P10.p die in the L2 stage. Upon gonad ablation in the L1 stage, P(5–8).p died at the same time as P9.p and P10.p (or shortly after), all in the L2 stage (Figure 1b and Table 1). Nine animals were followed through the L2 stage; in

Figure 1



Vulval development in *Panagrolaimus sp.* PS1732, *Turbatrix aceti* and *Halicephalobus sp.* (**a**) The vulva (V) is formed during the L3 and L4 larval stages from the four precursor cells P(5–8).p. These cells are born in the L1 stage and are aligned anteroposteriorly in the ventral cord. The anterior-most cells, P(1–4).p, die at birth (x) in all three species. In *Panagrolaimus sp.* PS1732, P(9–11).p fuse with the epidermal syncytium (s). In *T. aceti*, P(9,10).p apparently become neurons (n), on the basis of nuclear morphology, and P11.p fuses with the epidermal syncytium. In *Halicephalobus sp.* JB128, P11.p dies in the L1 stage, as do P(1–4).p, and P9.p and P10.p die in the L2 stage (X denotes death in the L2 or L3 stages, several hours after the cells are born). Filled circles designate cells which remain undifferentiated. Laser ablation of the gonad in the early L1 stage (**b**) causes P(5–8).p to adopt a non-vulval fate (S) in *Panagrolaimus sp.* PS1732; to die in the L3 stage in *T. aceti* (at the time when these cells normally divide); or to die in the L2 stage at the same time as P9.p and P10.p in *Halicephalobus sp.* JB128. Thus, the gonad sends an inductive signal in *Panagrolaimus*, but a survival signal in the other two species. In the latter case, specification of vulval fates might occur through the same, or another, gonadal signal. Alternatively, the survival signal might allow expression of an autonomous program of vulval specification.

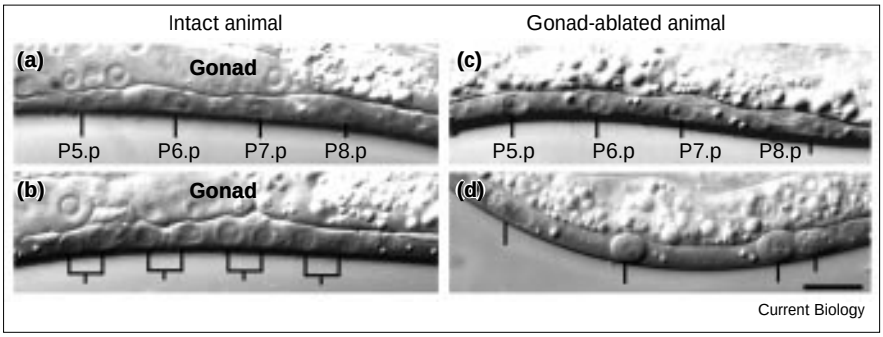
seven of the nine animals, P(5–8).p died in the L2 stage, at the same time or shortly after P9.p and P10.p; in the remaining two animals, P7.p survived until the L2 molt. If the gonad was ablated in the L2 stage, P(5–8).p survived (Table 1). Thus, the survival of P(5–8).p depends on a gonadal signal sent in the late L1 or early L2 stage.

A cell-survival signal in nematodes

These observations constitute the first demonstration of negative regulation of cell death by intercellular signals in nematodes. Most cell death in *C. elegans* occurs through the activation of a cell-autonomous death program [2], except for two instances in male development where a cell sends a death-inducing signal that kills another cell [9]. In contrast, death of the vulval precursor cells is prevented by intercellular signals in *Turbatrix* and *Halicephalobus*. Thus, not only is the intracellular cell-death machinery conserved from nematodes to vertebrates, the intercellular signaling that prevents cell death might also be conserved.

In vertebrates, survival signals act to select cells from among a population, for instance those that are correctly positioned [1,10]. Surprisingly, in *Turbatrix*, the L3-stage cell-death program is reproducibly repressed in the P(5–8).p cells during normal development and is not activated at this time in any other cells. The survival signal does not, therefore, appear to act selectively on some cells but not on others. It might, however, act selectively under more extreme circumstances: for instance, it is possible that, in some animals, a subset of the vulval precursor cells become mispositioned relative to the gonad. In some species of the same family (Panagrolaimidae), such mispositioned cells would adopt a vulval fate because in these species, upon gonad ablation, P(5–8).p adopt a

Figure 2



Death of the vulval precursor cells after gonad ablation in *Turbatrix aceti*. (**a**,**b**) No ablation. (a) Nomarski photomicrograph of the P(5–8).p cells in the L3 stage in an intact animal; (b) the same animal taken later, after P(5–8).p had undergone division. (**c**,**d**) Corresponding stages after ablation of the gonad in the L1 stage. (c) Morphology of the P(5–8).p cells in the L3 stage; (d) death of these cells slightly later in the same animal (refractile appearance). Bar = 10 μm. Anterior is to the left, ventral to the bottom.

Table 1

Gonad ablation in *Turbatrix aceti* and *Halicephalobus sp.* JB128.

Ablated cells	Time of ablation (stage)	Number of P(5–8).p cells adopting a vulval fate	Number of dying P(5–8).p cells
(a) ***Turbatrix aceti***			
None	–	All	None
Gonad	Early L1	1/92	91/92
Germ line only	Early L1	20/20	0/20
Gonad	Late L1 to early L2	2/20	18/20
Gonad	Late L2	26/28	2/28
(b) ***Halicephalobus sp.*** JB128			
None	–	All	None
Gonad	Early L1	2/104	102/104
Gonad	L2	36/36	0/36

The gonad was ablated at different times during development, and the effect of the ablation on the P(5–8).p cells assessed. Each of the four cells were scored in each animal, so the number of animals studied was one-quarter of the total number of cells. Ablation of all gonadal cells, or of somatic gonadal cells only, gave the same results.

vulval fate autonomously ([11]; our unpublished observations). In *Turbatrix*, the death of these cells through lack of a gonadal signal could prevent ectopic vulval differentiation and thereby prevent misalignment of vulval muscle or nerve cells [12] and development of a non-functional vulva.

In *Halicephalobus*, P(5–8).p die after gonad ablation in the L2 stage, at the same time as P9.p and P10.p die in the intact animal. It is therefore possible that, in this species, the survival signal does not reach P9.p and P10.p, but acts selectively on P(5–8).p. To test whether P9.p and P10.p could replace P(5–8).p, we ablated the precursor cells of P(5–8).p in the early L1 stage (before they migrate into the ventral cord and divide to give rise to the P(5–8).p cells). In all of the six animals that we studied, P9.p and P10.p could not replace the ablated vulval precursors. In this experiment, P9.p and P10.p did not move towards the gonad and thus might have failed to replace the more central vulval precursor cells. The gonad-derived survival signal might be redundant, and there might be another system specifying Pn.p fates along the anteroposterior axis, for instance one that relies on patterning through the differential expression of genes of the homeotic cluster (HOM-C). It is possible that, because of prior action of the HOM-C genes, the survival signal is not required for patterning in this species, but rather is only an evolutionary remnant of a patterning mechanism previously used in the history of this species. Alternatively, dependence on a survival signal could act to kill mispositioned cells within the P(5–8).p group, as proposed above for *T. aceti*.

What could be the molecular mechanism underlying the survival signal? In *Pristionchus pacificus* (family Diplogastridae), P(1–4).p and P(9–11).p die in the L1 stage [6], and death is repressed in P(5–8).p in the L1 stage by the activity of a HOM-C gene, *lin-39* [13], independent of the presence of the gonad [6]. In *C. elegans*, the *lin-39* gene is expressed in P(3–8).p from the L1 stage and prevents these cells from adopting a non-vulval epidermal fate irreversibly [14,15]. HOM-C genes also act in *C. elegans* at the time of vulval induction during the L3 stage and modulate competence of the P(3–8).p cells to respond to the inductive gonadal signal — the LIN-3 protein (related to epidermal growth factor) — emanating from the anchor cell [16]. Conversely, the gonadal signal enhances expression of *lin-39* in the closest vulval precursor cells through the Ras–MAP (mitogen-activated protein) kinase cascade [17]. Thus, at the molecular level, specification by the HOM-C genes and by the gonadal signal are linked in *C. elegans*. In *Turbatrix* and *Halicephalobus*, it is thus possible that the gonad-derived survival signal acts to activate expression of the *lin-39* homolog in P(5–8).p (as in *C. elegans*), thereby inhibiting the cell-death program (as in *P. pacificus*). Specification of Pn.p survival provides a good model for studying whether this simple rewiring of molecular pathways is involved in the formation of a cell-survival signal during evolution.

Materials and methods

T. aceti (vinegar eelworm), a gonochoristic species, was obtained from Carolina Biological Supplies. *Halicephalobus sp.* JB128, a parthenogenetic species, was a generous gift of J. Baldwin and C. Dolinski, and was identified by P. DeLey. They were handled using the techniques developed for *C. elegans* [18], and cultured on the *Escherichia coli* strain OP50 at 25°C. All nematodes mentioned in this study are in the family Panagrolaimidae, except *C. elegans* (family Rhabditidae) and *P. pacificus* (family Diplogastridae) [19]. Phylogenetic relationships among Panagrolaimidae are as yet unclear.

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